Tropical Journal of Pharmaceutical Research March 2015; 14 (3): 415-421
ISSN: 1596-5996 (print); 1596-9827 (electronic)

Available online at http://www.tjpr.org
http://dx.doi.org/10.4314/tjpr.v14i3.9

Original Research Article

In vitro Protective Effect of Ganoderol A Isolated from *Ganadermalucidum* Against Ultraviolet A Radiation and its Anti-inflammatory Properties

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Received: 17 April 2014 *Revised accepted: 16 January 2015*

Abstract

Purpose: *To evaluate the ultraviolet A (UVA) protection and anti-inflammatory activity of ganoderol A extracted from Ganodermalucidum.*
Methods: *The cytotoxicity and in vitro protective effect of ganoderol A against UVA damage were evaluated by MTT assay. Apoptosis and cell-cycle arrest of NIH/3T3 fibroblast cells were assayed by fluorescence-activated cell sorting (FCS). Expression of monocyte chemotactic protein-1 (MCP-1) and inducible nitric oxide synthase (iNOS) were determined using quantitative real-time polymerase chain reaction (qPCR).*
Results: *The results indicate that the maximal non-toxic concentration of ganoderol A in NIH/3T3 cells and RAW 264.7 macrophages was 50 and 25 µg/mL respectively. DNA in the tails and tail length decreased by 55 and 70 %, respectively, in the group pretreated with ganoderol A compared with the UVA-treated group. G1 phase cells decreased by 23 %, whereas the number of apoptotic cells returned to normal. The expression of MCP-1 and iNOS declined to 60 and 15 %, respectively, compared with LPS-stimulated group.*
Conclusion: *Ganoderol A has significant anti-inflammatory activity and protection against UVA damage, thus suggesting that the compound is a candidate for the development of a suitable product to protect skin from UV-induced photoaging.*

Keywords: *Anti-ultraviolet A, Anti-inflammatory, Ganoderol A, Ganodermalucidum, Photoaging*

Tropical Journal of Pharmaceutical Research is indexed by Science Citation Index (SciSearch), Scopus, International Pharmaceutical Abstract, Chemical Abstracts, Embase, Index Copernicus, EBSCO, African Index Medicus, JournalSeek, Journal Citation Reports/Science Edition, Directory of Open Access Journals (DOAJ), African Journal Online, Bioline International, Open-J-Gate and Pharmacy Abstracts

INTRODUCTION

Photoaging, a form of skin aging that is induced by chronic exposure to solar UV radiation, is the most critical factor in the pathogenesis of several skin diseases [1]. Clinically, the symptoms of photoaging in the skin manifest as mottled dyspigmentation, wrinkling, sagging of the skin, fragility, easy bruisability, loss of elasticity, accumulation of pre-cancerous lesions, and epithelial neoplasms [2].The reaction between ultraviolet rays and skin can cause damage via oxidative stress, inflammation, DNA damage, production of proteolytic enzyme, and immunosuppression[3]. It is well established that UVA participates in the chronic damage associated with photoaging and is considered as the "aging ray". Chronic exposure to UVA radiation can also lead to skin injury as well as malignancies [4]. New UVA-protective agents are

therefore urgently needed to provide increased skin protection.

Ganodermalucidum is a medicinal mushroom known in China as "Lingzhi." Its fruiting bodies have been used in traditional medicine for more than 2,000 years in China and many Asian countries [5]. *Ganodermalucidum* has been used in the treatment of hypertension, debility and weakness, cardiovascular disease, arthritis, bronchitis, neurasthenia, hepatopathy, insomnia, chronic hepatitis, gastric ulcer, nephritis, asthma, altitude sickness, diabetes, and tumors [6]. *Ganodermalucidum* is also effective in preventing oxidative damage, inflammation and resulting disease including skin aging [7]. Several active ingredients such as triterpenoids, polysaccharides, unsaturated fatty acids and ergosterol have been identified in *Ganodermalucidum* [8]. Ganoderol A is a terpenoid extracted from *Ganodermalucidum* and has been reported to exhibit antiviral and antimicrobial activities [9]. To our knowledge, however, UV-protective activity has not been reported for Ganoderol A. The objective of this study was to evaluate whether it would protect against UVA-induced skin damage.

EXPERIMENTAL

Chemicals

TRIzol® reagent, low-melting-point agar, and regular agar were obtained from Invitrogen (CA, USA). Fetal bovine serum (FBS), and Dulbecco's modified Eagle medium (DMEM) were bought from Gibco (CA, USA). Lipopolysaccharide (LPS), 2-(2,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT), propidium iodide (PI), and annexin V-FITC were purchased from Sigma-Aldrich (Sigma - Aldrich, USA). All other reagents used were of pharmaceutical grade.

Cell culture

NIH/3T3 fibroblast cells and RAW 264.7 macrophages were obtained from Cell Bank of the Chinese Academy of Sciences (Shanghai, PR China). Cells were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10 % heat-inactivated fetal bovine serum (FBS) and 100 U/mL penicillin/streptomycin in a humidified incubator with a 5 % CO_2 atmosphere at 37 °C.

Preparation of ganoderol A

Ganodermalucidum was ground into fine powder and passed through a 500 µm aperture size meshes then double-extracted by methanol (8:1). Two extracts were obtained which were termed extracts A and B, respectively. Extract A was obtained by removing methanol after filtration.

Partition

Extract A was extracted by water and ethyl acetate, the ethyl acetate part was collected, and ethyl acetate was removed by vacuum relief, then the extract B was obtained, and its weight was about 1.2 % that of extract A.

Extract B was separated via silica column chromatography, eluted by petroleum ether:ethyl acetate:formic acid (6:1:1), and were collected as ten fractions (marked 1 to 10) by one volume of the silica column. The column was eluted with petroleum ether:ethyl acetate:formic acid (5:1:1), and collected as twenty fractions (marked 1 to 20) by one volume of the silica column. Lastly, the column was eluted with petroleum ether:ethyl acetate:formic acid (10:10:1), and collected as thirty fractions (marked 1 to 30) by one volume of the silica column. The target compound was contained in the 29th fraction; after removing the solvent by vacuum relief, its purity was confirmed as 98 % by HPLC.

The target compound was identified as ganoderol A by comparison with a ganoderol A standard compound (PI & PI Biotech Inc).

Cytotoxicity assay

The cytotoxicity of ganoderol A toward NIH/3T3 fibroblast cells and RAW 264.7 macrophages was determined using 2-(2, 5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2H-tetrazolium bromide (MTT) assay. Cells were plated in 96-well plates in 100 µL growth media (DMEM + 10 % FBS), cultured overnight and exposed to a range of concentrations of ganoderol A for 48 h. A sample of 10 µl from a 5 mg/mL MTT stock solution was added to each well, and the plate was incubated for 4 h in darkness. The MTT solution was discarded and 100 µL dimethyl sulfoxide (DMSO) was added to each well. Plates were gently shaken for 15 min at room temperature. The optical densities (OD) of each well were measured with an enzyme immunoassay (EIA) (Bio-Rad USA) reader at 570 and 630 nm. The cell viability at each concentration of ganoderol A was determined by comparison with a ganoderol A-free control.

UVA irradiation protection test

NIH/3T3 fibroblast cells were grown in 96-well plates to 60–70 % confluence. The medium was

discarded and the growth media containing the indicated concentrations of ganoderol A were added to the plates. The plates were cultured at 37 °C in a humidified atmosphere with 5 % CO_2 for another 24 h. The medium was replaced with sterilized PBS and the plates were treated with UVA at 1.4 J/cm^2 for 45 min (50 % cytotoxic irradiation dose).The UVA irradiating source was a Philips Original Home Solarium (model HB 406/A, Philips, Groningen, Holland), and the radiation dose was measured with a UV Power Pack Radiometer (EIT Inc, Sterling, VA, USA). Following UVA exposure, PBS was removed and NIH/3T3 fibroblasts were replaced with the growth medium without ganoderol A. A control plate received UVA exposure without compound pretreatment. At 48 h after UVA irradiation, the cell viability of each group was measured by MTT assay as described above.

Comet assay

NIH/3T3 fibroblast cells were grown in 12-well plates to 60 – 70 % confluence. The medium was discarded and the growth medium containing 6.25 µg/mL ganoderol A was added to the plate at 1 mL/well. The plates were cultured at 37 °C in a humidified atmosphere with 5 % CO_2 for another 24 h, and were subsequently irradiated with 1.4 J/cm^2 UVA for 45 min. After 24 h the cells were digested and collected by centrifugation. Cells were suspended in PBS at a cell concentration of 1 × 10^6 cells/mL. Cell suspensions mixed with 0.8 % low-melting-point agar were placed onto a slide precoated with 0.7 % regular agar. After the agar solidified, slides were soaked in fresh, pre-chilled lysing solution (pH 10) for 1.5 – 2.0 h at 4 °C. After rinsing with 0.4 M Tris buffer (pH 7.5), slides were placed in a reservoir filled with fresh electrophoresis buffer (pH > 13) for 15 min and then subjected to electrophoresis for another 15 min at 25 V, and 300 mA. Slides were neutralized three times with Tris-HCl (pH 7.5), stained with PI and photographed under a fluorescent microscope (OLYMPUS IX71). The percentages of tail DNA and tail length, respectively, which indicate damaged DNA were analyzed using Casp software program (version 1.2.2, CaspLab, University of Wroclaw, Institute of Theoretical Physics).

Apoptosis and cell cycle assay

NIH/3T3 fibroblasts were cultured in a 6-well plate to 60 – 70 % confluence and treated with growth medium 6.25 µg/mL ganoderol A for 24 h. The cells were then exposed to 1.4 J/cm^2 UVA irradiation for 45 min. After another 24 h, the NIH/3T3 cells were harvested and washed twice with cold PBS. The cells were then re-suspended in 100 µL incubation buffer containing annexin V-FITC and PI. After being incubated in darkness for 15 min, apoptosis was analyzed by fluorescence activated cell sorting (FACS). For cell cycle analysis, the NIH/3T3 cells were harvested in cold PBS, fixed in 70 % ethanol, and stored overnight at 4 °C. The cells were washed once with PBS re-suspended in 1 mL 50 mg/mL PI staining reagent containing 100 µg/mL RNase, and then incubated in darkness for 30 min. The percentages of cells in each phase of the cell cycle were measured by FACS.

LPS-stimulated inflammation test

RAW 264.7 macrophages were cultured in 6-well plates to 60 – 70 % confluence. The medium was discarded and cells were washed twice with PBS. Serum-free DMEM medium was then added to each well. After 24 h, the medium was replaced with growth medium containing 6.25 µg/mL ganoderol A. The plates were incubated for 4 h and the medium was replaced by the LPS (100 ng/mL) induction medium. The plates were cultured at 37 °C in a humidified atmosphere with 5 % CO_2 for another 24 h and collected for total RNA isolation.

RNA isolation, reverse transcription, and quantitative real-time PCR (qPCR)

Total RNA was extracted using TRIzol® reagent, and RNA concentrations were measured using a spectrophotometer (Thermo, USA) at wavelengths of 260 and 280 nm. Extracted RNA (500 ng) was reverse-transcribed into cDNA using a PrimeScript RT reagent kit (Takara, Japan). The qPCR assays were conducted using SsoFast™ EvaGreen® Supermix (Bio-Rad, USA) according to the manufacturer's instructions. The primer pairs used were specific for iNOS (F: 5'-AAG CAG CTG GCC AAT GAG-3' and R: 5'-CCC CAT AGG AAA AGA CTG CA-3'), MCP-1 (F: 5'-GTC TCT GCA ACG CTT CTG TGC C-3' and R: 5'-AGT CGT GTG TTC TTG GGT TGT GG-3'), GAPDH (F: 5'-GTC ATT GAG AGC AAT GCC AG-3' and R: 5'-GTG TTC CTA CCC CCA ATG TG-3'). The relative expression of each gene was normalized to the housekeeping gene GAPDH.

Statistical analysis

Data were analyzed using GraphPad Prism 5 (GraphPad Software, version 5.01, La Jolla, CA, USA). Results are given as mean ± SEM and statistical significance was determined via Student's t-test. $P < 0.05$ was considered statistically significant.

RESULTS

Cytotoxicity and anti-UVA irradiation activity of ganoderol A

Ganoderol A was isolated from *Ganodermalucidum* and its structure was identified (Figure 1A). The cytotoxicity and cell-based anti-UVA damage activity on NIH/3T3 cells were determined by MTT assay. The maximal non-toxic concentration of ganoderol A on NIH/3T3 cells was 50 µg/mL (Figure 1B). The cell viability was upgraded 28 % when pretreated with 6.25 µg/mL ganoderol A. In contrast, when the concentration of ganoderol A exceeded 12.5 µg/mL, it significantly promoted the role of UV damage (Figure 1C).

Ganoderol A protects cells against UVA - induced DNA damage

Through a comet assay, we observed that UVA irradiation (1.4 J/cm^2, 45 min) caused severe DNA damage in NIH/3T3 cells, resulting in the appearance of broken DNA as the comet tails after single-cell gel electrophoresis. Pre-treatment with 6.25 µg/mL ganoderol A for 24 h greatly decreased the DNA lesion caused by UVA irradiations. The percentage of DNA in the tails and tail length decreased by approximately 55 and 70 %, respectively in the group pretreated with ganoderol A (Figure 2).

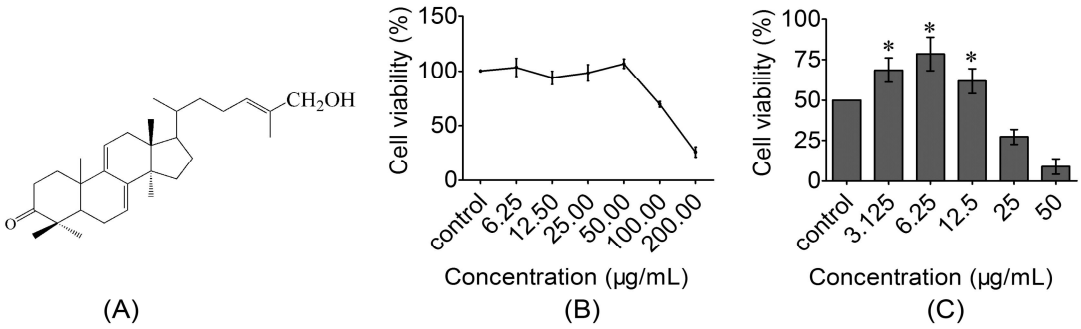

Figure 1: (A) Chemical structure of ganoderol A extracted from *Ganodermalucidum*;(B) Cytotoxicity; (C) *in vitro* activity of ganoderol A against UVA damage (1.4 J/cm^2, 45 min) in NIH/3T3 cells, determined by MTT assay; data are expressed as mean ± SEM (n = 3), **p* < 0.05 vs. control group

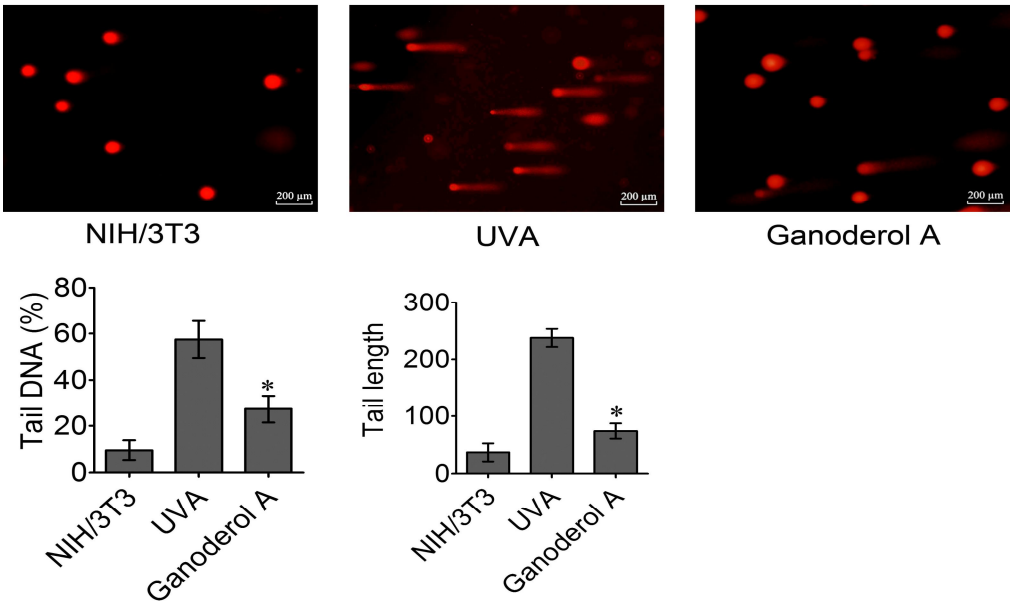

Figure 2: Protection by ganoderol A against DNA damage was analyzed by comet assay. The percentage of tail DNA and tail length which indicates damaged DNA, was analyzed using Casp software. Data are expressed as mean ± SEM (n = 3); **p* < 0.05 vs. UVA control group

Ganoderol A inhibits apoptosis and G1 phase arrest induced by UVA irradiation

After exposure to UVA for 24 h, the NIH/3T3 cells were stained with annexin-V and PI and the degrees of apoptosis were assayed by flow cytometry. As shown in Figure 3, the proportion of apoptotic cells increased by 8.76 % after low dose UVA irradiation, whereas the proportion of apoptotic cells returned to normal in NIH/3T3 cells pretreated with ganoderol A. The percentage of G1 phase increased about 32 % after UVA-irradiation but after pretreatment with ganoderol A, the proportion of G1 phase decreased 13 % compared with the UVA group. These results demonstrate that ganoderol A could protect cells from apoptosis and G1 phase arrest induced by UVA irradiation.

Anti-inflammatory effect of ganoderol A on LPS-challenged RAW 264.7 macrophages

The maximum non–toxic concentration of ganoderol A on RAW 264.7 macrophages was 25 μg/mL (Figure 4A). Pre-treatment with ganoderol A significantly inhibited the production of monocyte chemotactic protein-1 (MCP-1) and inducible nitric oxide synthase (iNOS) in LPS-activated RAW 264.7 macrophages. The expression of MCP-1 (Figure 4B) and iNOS (Figure 4C) dropped to 60 and 15 %, respectively, compared with LPS-stimulated group. However, ganoderol A did not have significant effects on the expression of additional inflammatory cytokines such as IL-1, IL-6 and TNF–α in LPS-activated macrophages (data not shown).

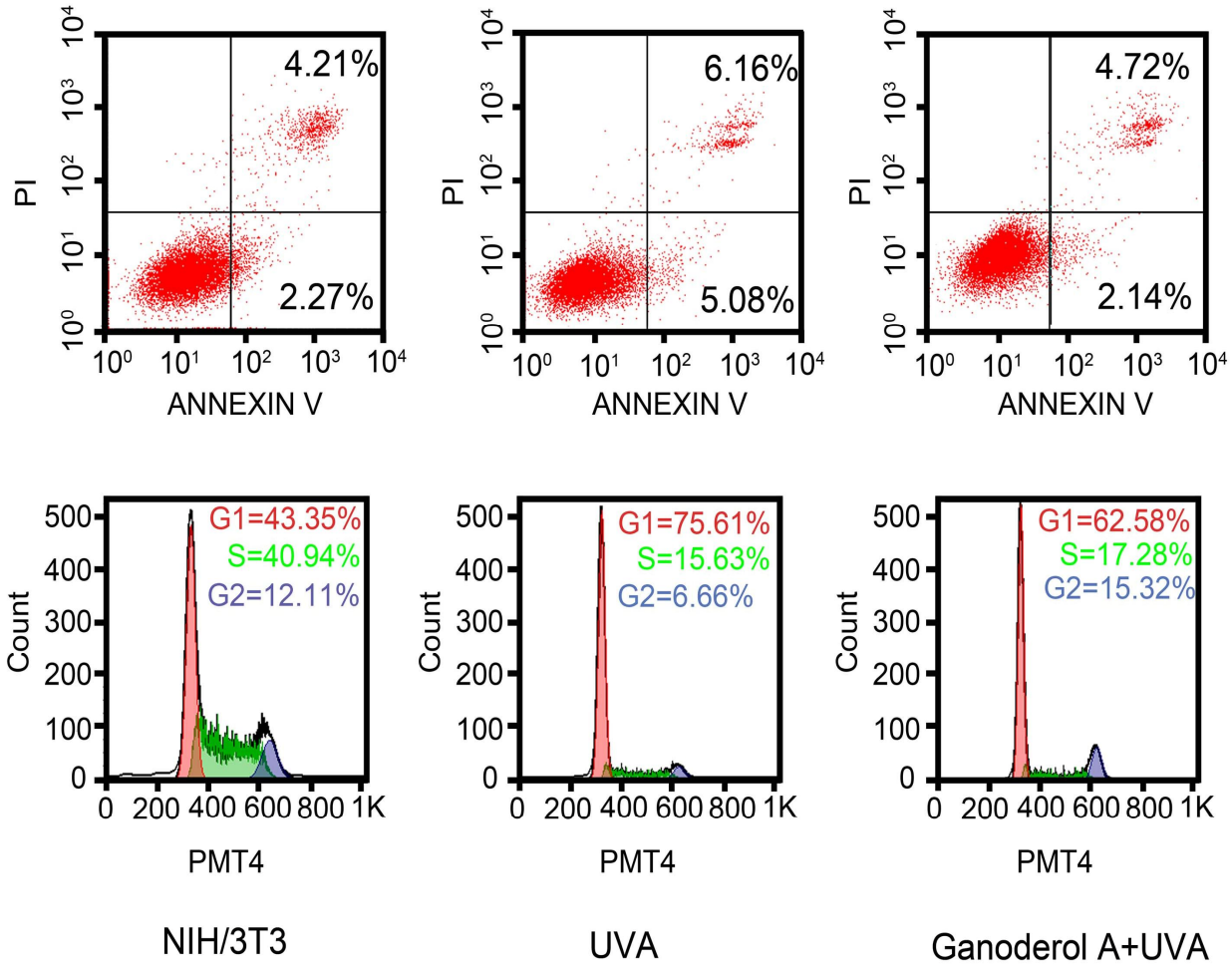

Figure 3: Inhibitory effects of ganoderol A on cell apoptosis and cell-cycle arrest induced by UVA irradiation 24 h after exposure to UVA (1.4 J/cm^2, 45 min). NIH/3T3 control group, UVA-irradiated cell group, and the group pretreated with ganoderol A (6.25 μg/mL) were stained with annexin-V and PI and the cell apoptosis was assayed by FACS. For cell-cycle assay, the cells were stained with PI staining reagent and percentage of cells in each phase of the cell cycle was measured by FACS

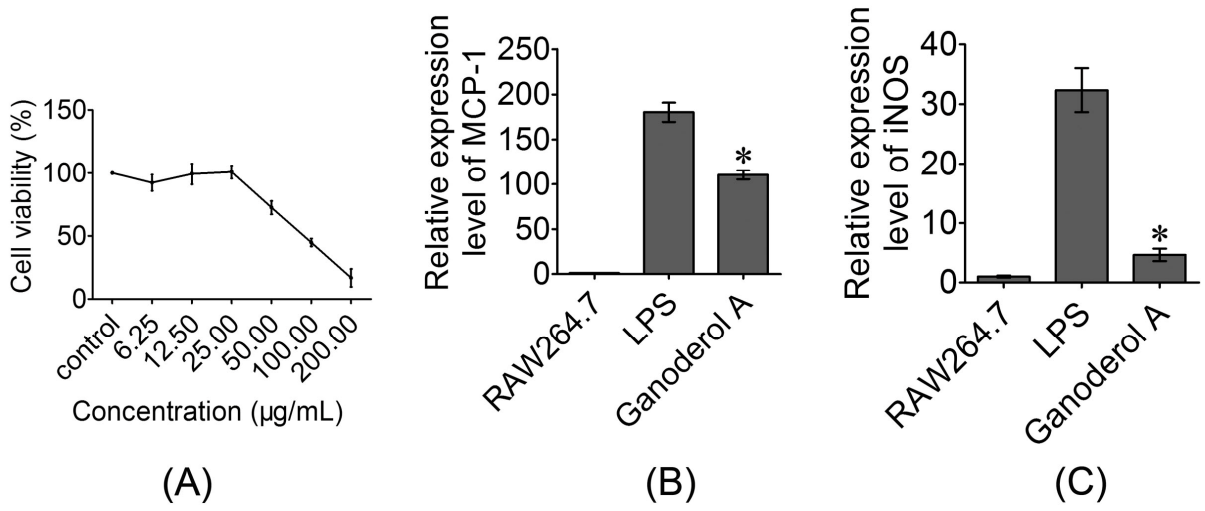

Figure 4: Effect of ganoderol A on the expression of inflammatory cytokines in LPS-activated RAW 264.7 macrophages. (A) Cytotoxicity of ganoderol A on RAW 264.7 macrophages was measured *via* MTT assay. Relative mRNA expression levels of MCP-1 (B) and iNOS (C) compared with the LPS group were analyzed by qPCR. Data are expressed as mean ± SEM; *$p < 0.05$ vs. LPS group

DISCUSSION

The skin is the largest organ of the body, and protects the body by acting as a physiological barrier against the damaging effects of ultraviolet rays. Photoaging, which is caused by chronic exposure to solar radiation, is a complicated biological snowballing process and affects diverse constituents of skin [1]. The use of chemoprotective agents, especially natural products such as plant polyphenols, has been reported to provide significant photoprotective effects, thereby inhibiting damage to skin exposed to UV radiation [4]. In the present study, it was shown that ganoderol A might be an effective photoprotective agentagainst UVA-irradiation induced DNA damage, apoptosis, cell-cycle arrest and inflammatory skin damages.

It is well documented that 50 % of total solar UVA radiation penetrates the dermis [10]. UVA photons exert most damage on skin cells in an oxygen-dependent manner and on a broad range of cellular targets, including proteins, membranes, and DNA [11]. Longwave UVA radiation is not directly absorbed by DNA. The genotoxicity of UVA is most likely induced by reactive oxygen species (ROS) including superoxide anion (O_2^-), hydroxyl (OH^-), and hydrogen peroxide (H_2O_2) via indirect mechanisms [12]. It has been well described that when cells are exposed to UVA irradiation the cell cycle is arrested in G1 phage to facilitate DNA repair in order to protect cells against mutagenesis [13]. Direct damage, ROS, and lipid peroxidation induced by UVA can induce cell apoptosis [14]. Previous studies have further reported that inflammation is related to ultraviolet-radiation-induced protein denaturation, DNA damage and oxidative stress [15]. In this study, we showed that, following low dose UVA irradiation (1.4 J/cm^2, 45 min), NIH/3T3 cells exhibited significant cell cytotoxicity including DNA damage, apoptosis, and cell-cycle arrest. Pretreatment with ganoderol A (6.25 μg/mL) before UVA irradiation significantly inhibited these forms of damage caused by UVA irradiation. Macrophages are known to be one of the acute immune cells in the regulation of inflammatory responses. Activated macrophages secrete a number of inflammatory mediators [16]. In LPS-challenged RAW 264.7 macrophages, we found that ganoderol A could greatly inhibit the expression of LPS-induced pro-inflammatory molecules, including MCP-1 and iNOS.

Over the past decades, studies have revealed that ROS are the main inducing factors for skin damage resulting from UVA irradiation [17]. Continued ROS-induced oxidative stress can activate a variety of transcription factors such as NF-κB, p53 [18]. These activated transcription factors can modulate the expression of inflammatory cytokines, inducing cell-cycle arrest and apoptosis as well as oxidative stress [19]. Chronic inflammation can stimulate the process of oxidative stress by secreting a series of inflammatory cytokines such as iNOS. Many natural products such as plant polyphenols and terpenoids strongly modulate the oxidative stress signaling, such as NF-κB, p53, and mitogen-activated protein kinase (MAPK) signaling pathways [5]. Further research is required to confirm whether the anti-UVA and anti-inflammatory activities exhibited by ganoderol A

are also manifested following the modulation of these oxidative stress signals.

CONCLUSION

The findings of this study show that ganoderol A can significantly attenuate cell damages induced by UVA irradiation, including DNA damage, apoptosis, and G1 phase cell-cycle arrest. It also exhibited efficient anti-inflammatory property by downregulating the expression of inflammatory cytokines MCP-1 and iNOS in LPS-sitimulated RAW264.7 macrophages. The low cytotoxicity and strong activity of ganoderol A suggest that it can effectively protect against UVA and might represent a promising, safer chemical additive in sunscreens.

ACKNOWLEDGEMENT

This work was supported by Key Projects in the National Science & Technology Pillar Program during the Twelfth Five-year Plan Period (no. SQ2011SF12B02099), Guangzhou Science and Technology Plan Projects (no. 2013J4500058), and Guangdong University Scientific and Technological Achievements of Major Projects (no. cgzhzd0905).

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